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Influence of dietary supplementation with flaxseed and *lactobacilli* on the cells of local innate immunity response in the jejunal mucosa in piglets after weaning

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ABSTRACT

A histological study was designed to determine the influence of flaxseed and/or *lactobacilli* inclusion in the diet of piglets from 10 days before to 21 days after weaning. The selected inflammatory cell population incidence in the piglet jejunal mucosa was investigated. Significantly higher numbers of myeloperoxidase-positive ($P<0.01$) and CD163-positive ($P<0.001$) cells in the jejunal mucosa were recorded on the weaning day and for 7 days after ($P<0.001$ and $P<0.01$, respectively) in the flaxseed group compared with the basal diet. The number of intraepithelial lymphocytes was also significantly increased until 3 days after weaning ($P<0.001$). A prolonged significant increase in the myeloperoxidase-positive cells and intraepithelial lymphocyte numbers in the flaxseed + *lactobacilli* group was detected. In contrast, the number of CD163-positive cells in the flaxseed + *lactobacilli* group was significantly lower on the day of weaning ($P<0.05$) and 3 days after ($P<0.01$). The same effect was observed in the group with *lactobacilli* alone during the first 3 days after weaning ($P<0.05$ and $P<0.01$, respectively) and these findings indicate down-regulation of CD163 expression in the jejunal mucosa by *lactobacilli*. The presence of *lactobacilli* in the diet had a stimulatory effect on goblet cell quantity in the epithelium ($P<0.001$) and a distinct 50% reduction in the flaxseed group ($P<0.01$) compared with the basal diet was observed on the weaning day. A significant increase in myeloperoxidase-positive cell number in the jejunal mucosa in the flaxseed + *lactobacilli* group was the only significant difference ($P<0.05$ and $P<0.01$, respectively) found 21 days after weaning in comparison with all the other groups, indicating the pro-inflammatory effect of this feed additive combination. We conclude that dietary supplementation with flaxseed and *lactobacilli* on the cells of local innate immunity response in the jejunal mucosa in piglets after weaning might be linked with significant anti-inflammatory effects in the jejunal mucosa.

Introduction

At weaning, piglets are subjected to many stressful circumstances associated with their change of environment, separation from the mothers, transport, and general replacement of fluid milk nutrition by solid food with a different composition and nutrient value. The complexes of these social, environmental and dietary stresses interfere with gut development and adaptation and are definitely responsible for maldigestion, malabsorption, shift in gut microflora, poor performance and diarrhoea in piglets after weaning (PW) (Verdonk, 2006). The pathophysiological base of all these disorders is closely connected with the inflammatory response of the intestinal mucosa.

The intestinal surface is covered by a protective mucous layer which represents the front line of innate host defence. The goblet cells (GC), which reside throughout the length of the intestine within the intestinal epithelial layer, are responsible for the

Abbreviations: F, flaxseed; FL, probiotic cheeses + flaxseed; GC, goblet cells; IEL, intraepithelial lymphocytes; MPO, myeloperoxidase; PUFA, polyunsaturated fatty acids; PW, post-weaning; S, sunflower oil; SL, probiotic cheeses + sunflower oil; WD, weaning day.

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production and maintenance of the mucous layer coating the gastrointestinal tract. Another special cell type present in the epithelium that is involved in the immunological interrelations are the intraepithelial lymphocytes (IEL), which make up the first immune cell line of defence in the intestine (Beagley and Husband, 1998). The majority of these cells are CD8-positive T cells (Ishikawa et al., 2007). Their specific function is not clear, but it has been suggested that they may play a variety of roles, such as immunoregulation and promoting epithelial damage repair (Shen, 2009). Among the connective tissue cells involved in the innate immunity inflammatory processes, a remarkable position is held by polymorphonuclear leukocytes and tissue macrophages. The tissue myeloperoxidase (MPO) activity is one of the earliest consequences of neutrophil inflammation (Nauseef, 2007), while in the absence of inflammation, MPO is not found in the extravascular tissue. The plasma membrane glycoprotein CD163, a member of the scavenger receptor cysteine-rich superfamily class B, is highly expressed on resident tissue macrophages and to a lesser extent on monocytes (Fabriek et al., 2005). The evaluation of MPO-positivity, CD163-positivity and numbers of IEL and GC was used in this study to assess the intensity of the inflammatory response. Many nutritional strategies are arranged to prevent economic losses connected with malnutrition of piglets PW. From a dietary point of view, various biologically active substances, e.g., probiotics, prebiotics, non-specific substrates, plant extracts, organic acids or polyunsaturated fatty acids, are included in the diet of piglets around weaning to maintain intestinal homoeostasis (Bomba et al., 2006). The aim of our experimental study was to analyse changes in the inflammatory cell populations present in the mucosa, which reflect the health status and the level of local innate immunity of the intestine and have a potential influence on diarrhoeic syndrome progress. The selected cell populations were recorded from the day of weaning (WD) to 21 days PW in piglets. Our objectives were: (1) to evaluate changes in the mucous-producing GC number in the jejunal epithelium; (2) to analyse the mucosal cells participating in the inflammatory response – IEL, MPO-positive cells and CD163-positive cells; (3) to evaluate the influence of biologically active substances on these processes – *lactobacilli* and/or flaxseed as a source of polyunsaturated fatty acids (n-3 PUFA); (4) to compare the differences between the experimental groups with different diets at the same time period as well as the time-dependent changes in the individual groups during 21 days PW.

Materials and methods

The State Veterinary and Food Administration of the Slovak Republic approved the experimental protocol number 2108/07-221 and the animals were handled and sacrificed in a humane manner in accordance with the guidelines established by the respective commission.

Probiotic bacteria

The *Lactobacillus* probiotic strains were isolated at University of Veterinary Medicine and Pharmacy, Košice, Slovakia. The *Lactobacillus plantarum* – Biocenol™ LP96 (CCM 7512) strain was selected from the gut content of a healthy suckling piglet and *Lactobacillus fermentum* – Biocenol™ LF99 (CCM 7514) was isolated from the gastrointestinal tract of an adult chicken. Cheddar cheese was used as a vehicle for probiotic strains at 1×10^9 CFU/g of cheese. The control cheese did not contain probiotic strains.

Animals, housing and diets

The experiment was carried out on 88 clinically healthy piglets (cross-bred – Yorkshire × Pietrain). The piglets were housed in typical indoor pens and were divided into four groups: control group S (control cheese + sunflower oil) 19 piglets; group SL (probiotic cheeses + sunflower oil) 23 piglets; group F (control cheese + whole crushed flaxseed) 23 piglets; group FL (probiotic cheeses + whole crushed flaxseed) 23 piglets.

Table 1
Fatty acid compositions (percentage) of flaxseed oil, sunflower oil and feed mixtures.

Fatty acid	Flaxseed oil	Sunflower oil	Feed mixtures
Lipids	45.78	ND	2.19
Palmitic. C16:0	5.1	6.3	17.4
Stearic. C18:0	3.7	3.2	2.2
Oleic. C18:1	18.4	22.6	24.7
Linoleic. C18:2	16.1	67.9	51.9
Linolenic. C18:3	56.8	0	3.8

ND, not detected.

The animals were fed OŠ-02 (Spišské kŕmne zmesi, Spišské Vlachy, Slovak Republic) feed mixture for early piglet weaning. The basal diet was supplemented with whole crushed flaxseed cultivar Flanders (Agrola Kožušice, Czech Republic): 100 g flaxseed (i.e., 45.8 g lipids)/1 kg basal diet for the F and FL groups and control oil: 45.8 g sunflower oil/1 kg basal diet for the S and SL groups. Table 1 gives the fatty acid profile of flaxseed, sunflower oil and the basal diet.

The piglets were weaned at the age of 28 days. Ten days before WD and 21 days PW the experimental piglets in groups SL and FL were supplied with probiotic cheeses daily (4 g/animal/day for each cheese). Piglets in the S and F groups were supplied with control cheese at a dose of 8 g/animal/day. The probiotic and control cheeses were supplied to piglets once a day (individually in the morning) in grated form on the surface of the feed. The animals liked the cheese and consumed it instantaneously. In the same period the animals had *ad libitum* access to water and feed supplemented with whole crushed flaxseed or sunflower oil.

Biological material

The pigs in all groups were sacrificed using T61 (Intervet International B.V., Boxmeer, The Netherlands, dose: 0.3 ml/kg body weight) intracardially on WD (28-day-old piglets) and on Days 3, 7 and 21 PW. The gastrointestinal tract was immediately removed from the sacrificed piglets. Small intestine samples (jejunum) 1–2 cm long were taken 10 cm from the Ligament of Trietz, washed with cold saline and fixed in 4% paraformaldehyde. The tissues were then embedded in paraffin wax, cut into 4–5 μm sections, and mounted. After deparaffinisation, the tissues were stained with haematoxylin–eosin (HE) for histological examination.

Histological cytoanalysis

For immunohistochemistry, wax embedded sections (4–5 μm thick) were deparaffinised and rehydrated. Endogenous peroxidase activity was blocked with 3% H_2O_2 with methanol. Pre-treatment was performed in a microwave oven at 600 W for 15 min in 0.01 M citrate buffer at pH 6.0. A primary anti-MPO rabbit polyclonal antibody (RB-373-A0; 1:100; Thermo Scientific, Waltham, MA, USA), and a primary anti-CD163 rabbit clonal antibody (DB 045-0.5; 1:100; DB-Biotech, Košice, Slovakia) were used. Primary antibodies were labelled using a two-stage indirect immunoperoxidase technique. Primary antibodies were applied at the appropriate titre (Table 2). Anti-mouse IgG (H + L)/anti-rabbit IgG (H + L) was used for detection of MPO-positive cells (TL-015-HDS; Thermo Scientific-Ultra Vision LPValue Detection System HRP Polymer & DAB Plus Chromogen; Thermo Scientific, Waltham, MA, USA). Biotinylated secondary goat anti-mouse IgG/goat anti-rabbit IgG (DAB150; Millipore IHC Select® Immunoperoxidase Secondary